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PRINCIPAL INVESTIGATOR: Darryl L. Hadsell, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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13. ABSTRACT (Maximum 200 Words) The hypothesis tested in this proposal is that overexpression of USF in the mammary glands of transgenic mice will inhibit myc-dependent tumorigenesis. To test this hypothesis, a transgene was constructed to target the overexpression of FLAG-tagged USF-2 to the mammary glands of virgin transgenic mice under the control of the mouse mammary tumor virus (MMTV) long terminal repeat. The transgene was evaluated in transfected cells in culture, and then injected into fertilized FVB mouse embryos. A total of 370 embryos were injected and transferred into recipient females resulting in 11 pregnancies. From these a total of 84 offspring were obtained, 12 of which were positive for the transgene. Females from the F ₀ and F ₁ generations of each line were screened for transgene expression by western blotting with an antibody specific for the FLAG peptide. Detectable expression was observed in a mammary gland from a single lactating F ₀ female. However, no expression was observed in any of the nonlactating virgin female F ₁ mice tested. In addition the single F ₀ female that was positive for transgene expression during lactation has thus far failed to produce viable offspring. This report discusses the implications of this data and suggests possible alternatives for testing the above hypothesis.				
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Introduction

Upstream stimulatory factor (USF) consists of two helix-loop-helix/zipper (bHLH/zip) proteins, USF-1 and -2, which are highly conserved among species and related to *c-myc* (1) transcription factors. Previously published cell culture studies with cancer cell models show that USF is both antiproliferative and can antagonize *c-myc* (2). The research described in this proposal addresses the idea that expression/activity of USF is a determining factor in tumor initiation and/or growth. This idea was to be explored by testing the hypothesis that targeted overexpression of USF-1 in the mammary glands of MMTV-*myc* transgenic mice will cause withdrawal from the cell cycle and differentiation thereby preventing tumors. The overall approach was to make and characterize transgenic mice that overexpress USF-1 under the control of the mouse mammary tumor virus long terminal repeat. Once in hand this new line of transgenic mice would be crossed with a previously described line of transgenic mice that overexpress *c-myc* in the mammary gland. A decreased in tumor frequency and/or an increase in tumor latency among mice that carry both MMTV-*myc* and MMTV-USF-1 as compare with those which just carry MMTV-*myc* would confirm the hypothesis.

Body

The approved statement of work for this project described two specific tasks to be completed over a 36-month period. The first task was to determine the effect of mammary-specific USF-1 overexpression on mammary gland development and lactation. This was to be completed during months 1 through 24. The second task was to determine the ability of mammary-specific overexpression of USF-1 to prevent *myc*-induced mammary tumors. This task was to be completed during months 9 through 36.

the major focus of task 1 for the first 12 months of the funding period was to construct transgene which overexpresses USF-1 in the mammary gland, use this transgene to produce transgenic mice, and then screen the resulting mice to identify 1 or 2 lines with biologically significant expression levels for use throughout the remainder of the project. During the first four months of the funding period, a transgene was constructed that was designed to target the expression of a tagged form of USF-2 to the virgin mammary gland under the control of the mouse mammary tumor virus long terminal repeat. Early in the period a decision was made to overexpress USF-2 as opposed to USF-1. This change was made because USF-2 was shown to display stronger antiproliferative as well as stronger *myc* antagonist activity than USF-1 (2). Consequently, USF-2 would be predicted show greater efficacy in the transgene model than USF-1. The overall design of the MMTV-USF-2 construct is presented in figure 1. The construct was produced from 2 starting plasmids, PN4 (2) and MMTV-SV40 (3) . Polymerase chain reaction was used to place a consensus Kozak (4) sequence and coding DNA for a FLAG peptide at the 5' end of the USF-2 cDNA. The primers used in this amplification also included unique restriction sites to facilitate subcloning. To produce the MMTV-USF-2 transgene, the resulting PCR product was then subcloned as a HindIII/EcoRI fragment into the analogous sites of the MMTV-SV40 expression vector. The resulting plasmid was then sequenced to confirm the accuracy of the open reading frame for USF-2. Following the confirmation of the correct coding sequence the USF-2 transgene, the USF-2 coding sequence was subcloned into a general-purpose expression vector, pSG5. This was used to evaluate that FLAG-tagged USF-2 in cultured cells. The

ability of the FLAG-tagged transgene protein to be detected by immunohistochemistry was confirmed in transfected HeLa cells in culture (Figure 1 C). This result confirmed that the modified USF-2 cDNA was capable of producing a protein. To address the biological properties of the FLAG-tagged USF-2 protein, colony formation during G418 selection was measured in cells transfected with either the parental USF-2 expression vector, the FLAG-tagged USF-2 vector, or an empty expression vector, pSG5 (Figure 2).

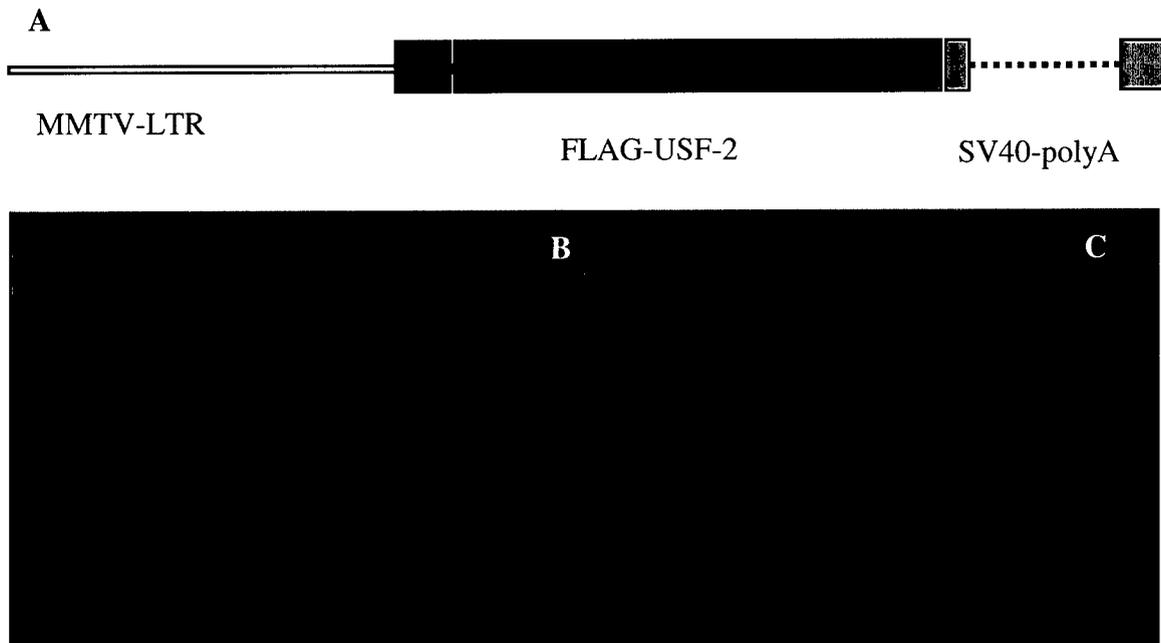


Figure 1. Design of the MMTV-USF-2 transgene (A) and immunofluorescent detection of FLAG-tagged USF-2 in transiently transfected HeLa cells (C). DAPI staining (blue) was used to detect both nontransfected and transfected cells (B). The transgene construct is driven by the MMTV-LTR (□) and contains coding sequence for the eight-residue FLAG peptide (■) fused in-frame to the N-terminal coding sequence for USF-2 (■). Sequence from SV40 T/t antigen provide an intron and poly A signal (■). FLAG-tagged USF-2 expression (red) is visible in 4 of the 8 cells present in the field.

This type of assay was originally used to demonstrate the antiproliferative effects of USF-2 (2) in tumor cells. Mammary epithelial cells or HeLa cells were transfected with one of the three vectors and a neomycin resistance vector. Following transfection the cells were selected in G418 for 1 month. After the selection period colony number was measured. Both the parental vector and the FLAG-tagged USF-2 inhibited colony formation in both HeLa cells and HC11 mammary epithelial cells. This supported the conclusion that the modified USF-2 was in fact capable of eliciting a biological response similar to the parental, unmodified USF-2. Following this evaluation the MMTV-USF-2 transgene was purified away from bacterial plasmid DNA by digestion with BssHII and preparative agarose gel electrophoresis. The resulting transgene fragment was then

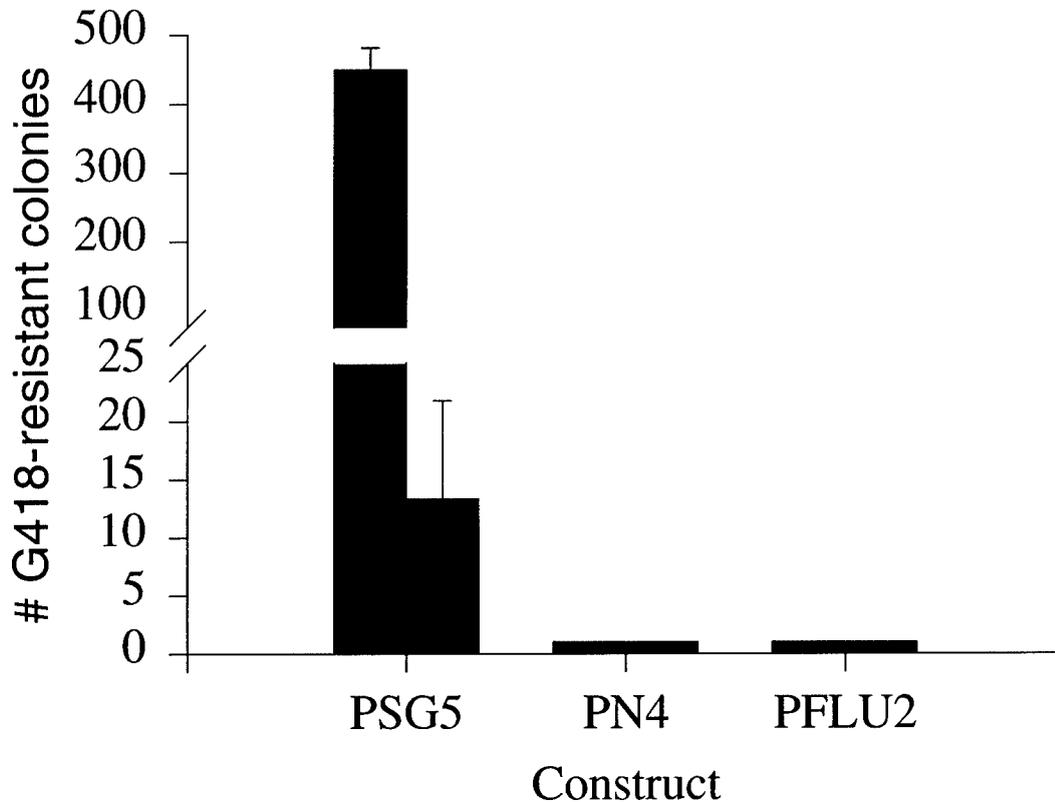


Figure 2. Inhibition of colony formation by USF-2 or a FLAG-tagged USF-2. Either HeLa cells (■) or HC11 mammary cells (■) were transfected with a neomycin resistance plasmid and either the empty expression vector (pSG5), the parental USF-2 expression vector (PN4), or the FLAG-tagged USF-2 expression vector (PFLU2). Colonies were then counted after a 4 weeks selection period in G418. Each bar represents the mean±SD. of three plates. Cells transfected with either PN4 or PFU2 had zero colonies after 4 weeks of selection in G418.

injected in FVB mouse embryos for the production of founder transgenic mice. The injection data is summarized in table 1. From four independent rounds of microinjection a total of 12 founders were obtained. Of these, 8 transmitted the transgene to F1 offspring, 2 were fertile, but did not transmit the transgene, and 2 were incapable of reproducing. Expression of the transgene in the mammary gland was initially evaluated by western blot analysis of total tissue lysates using the M2-2 monoclonal antibody to the FLAG epitope. This analysis has thus far produced mixed results (Figure 3). As illustrated in figure 3, the transgene protein can easily be detected when transiently transfected into HeLa cells (lane 1) In addition a band of the correct size has been detected in a single extract prepared from lactating mammary tissue of one of the founder females (lane 2). This band was not present in an extract prepared from a lactating nontransgenic sibling (lane 3). This supports the conclusion that the transgene protein was in fact expressed at a detectable

Table 1. Production of transgenic mice with the MMTV-USF-2 transgene by microinjection of fertilized one-cell FVB embryos.

Injection	Embryos injected	Offspring obtained	Positive offspring	F1 lines
1	118	34	0	0
2	99	25	1	0
3	94	9	4	3/4
4	59	16	7	5/7

level in a single founder female. Unfortunately, this founder has been incapable of producing any viable offspring to date. The remaining lines have, to date, been screened as virgin females (age 6 to 8 wks). This was because expression of the transgene in the nonlactating virgin gland would be optimal for successful completion of the proposed studies. Examples of the results from this screening are illustrated in lanes 4 through 13 of figure 3. To date, none of the eight lines screened by this procedure show detectable expression of the transgene protein in nonlactating virgin females.

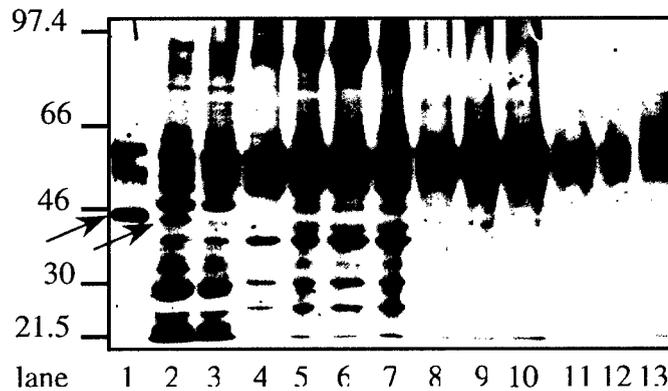


Figure 3. Western blot analysis for FLAG-tagged USF-2 in mammary tissue from transgenic and nontransgenic F1 females. Transiently transfected HeLa cells serve as a positive control (lane 1). The expected size of the protein is 44 kDa. Lanes 2 and 3 contain mammary tissue extracts from a lactating transgenic founder and her nontransgenic sibling respectively. Lanes 4, 8, 11 and 12 contain mammary tissue extracts from nonlactating F1 nontransgenic sibling females. Lanes 5,6,7 9,10, and 13 contain mammary tissue extracts from nonlactating F1 transgenic females. The red arrows highlight FLAG-tagged USF in both the positive control sample and a single lactating founder female. The numbers on the left side of the figure indicate molecular weight in kiloDaltons.

Key Research Accomplishments

- Constructed a transgene (MMTV-USF-2) designed to target USF-2 expression to the virgin mouse mammary gland.
- Validated the functionality of MMTV-USF-2 in cell culture
- Injected mouse embryos with MMTV-USF-2
- Obtained eight independent transgenic mouse lines that carry MMTV-USF-2.

Reportable Outcomes

- Development of a transgenic mouse model that may express USF-2 in the mammary gland during lactation.

Conclusions

To date eight lines of transgenic mice have been screened for expression by western blotting. The data obtained from these studies suggests that none of these lines display detectable expression levels in the virgin mammary gland. This inability to detect transgene expression may be due in part to limited sensitivity of the screening assay. A second possible reason of the apparent lack of expression could in fact be a true lack of expression. To address this we plan to rescreen both lactating and nonlactating females from the 8 lines by both RPA and by western blotting with an antibody to USF-2 protein. This approach will provide three critical observations: 1) the RPA is highly sensitive and will determine if low-level transgene expression occurs in these lines, 2) the western against USF-2 should detect both endogenous and transgene proteins and therefore would allow us to determine if the level of USF-2 is significantly greater in the transgenic than in the nontransgenic mice 3) biologically significant levels of expression observed during lactation, would allow the use the existing lines to complete task 2 in the original statement of work. If a line is obtained that displays an USF expression that is greater than two fold over that of nontransgenic mice then the studies described under task 2 will be conducted with this line.

As mentioned in the original proposal, a major limitation to the studies is the potential for failure of the transgene to express at detectable or biologically significant levels. This limitation, if it occurs would preclude the completion of the originally proposed studies. Therefore, alternative models must be considered in an attempt to test the original hypothesis that USF expression determines the risk for development of breast tumors. There are two possible alternatives that could be used to overcome the current limitation. The first, is the design of a new targeting construct and the screening and development of new lines of transgenic mice based on this construct. The second, is the testing of the hypothesis in mice that have reduced as opposed elevated expression of USF -2. There are currently lines of mice in existence which carry a targeted mutation in the gene for USF-2 (5-7). Studies on these lines have shown that loss of USF-2 results in a significant overall reduction in the abundance of the USF-1, a decrease in body weight, and alterations in hepatic gene expression. Our own studies with one of these lines of mice have found the loss of USF-2 diminishes lactational capacity. This diminishment is not associated with changes in mammary development during pregnancy. However,

there appears to be mild reductions in mammary ductal development prior to pregnancy. These results support the idea that the analysis of tumorigenesis in a USF-2 knockout model could be a viable alternative to the originally proposed approach. A revised statement of work is being prepared which will suggest alternative experiments to that proposed in task 2. These alternatives are: 1) determine the impact of loss of USF-2 on DMBA-induced tumorigenesis, 2) determine the impact of loss of USF-2 on myc-induced tumorigenesis.

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Appendices

None